RECOMBINANT FRAGMENTS OF THE HUMAN ACETYLCHOLINE RECEPTOR AND THEIR USE FOR TREATMENT OF MYASTHENIA GRAVIS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application is a continuation-in-part of application no. 09/423,398, filed November 8, 1999, as a 371 national stage application of PCT/IL98/00211, filed May 6, 1998, the entire contents of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Field of the invention

[0002] The present invention relates to polypeptides capable of modulating the autoimmune response to acetylcholine receptor, and more particularly to polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor α-subunit, which polypeptides are useful in the diagnosis and treatment of myasthenia gravis, and to DNA molecules encoding said polypeptides.

[0003] **ABBREVIATIONS:** **AChR** - acetylcholine receptor; **α-BTX** - **α**-bungarotoxin; **EAMG** - experimental autoimmune myasthenia gravis; **GST** - glutathione S-transferase; **hAChR** - human

acetylcholine receptor; **MG** - myasthenia gravis; **LNC** - lymph node cells; **MIR** - main immunogenic region.

Description of the Related Art

[0004] Myasthenia gravis (MG) is a human autoimmune disorder characterized by muscle weakness and fatigability. In this disease, antibodies against the acetylcholine receptor (AChR) bind to the receptor and interfere with the transmission of signals from nerve to muscle at the neuromuscular junction (Patrick and Lindstrom, 1973).

[0005] The acetylcholine receptor molecule is a transmembrane glycoprotein consisting of five homologous subunits, organized in a barrel-staves-like structure around a central cation channel, in the stoichiometry of either $\alpha 2\beta\varepsilon\delta$ in fetal, or $\alpha 2\beta\varepsilon\delta$ in mature, muscle (Karlin, 1980; Changeux et al., 1984). Noda et al. (1983) described the cloning and sequence analysis of human genomic DNA encoding the α-subunit precursor of muscle acetylcholine receptor, and Schoepfer et al. (1988) reported the cloning of the α-subunit cDNA from the human cell line TE671. Human muscle AChR α-subunit exists in two forms, one of which has 25 additional amino acid residues, inserted between positions 58 and 59, that are coded by the 75bp exon p3A (Beeson et al., 1990). The α-subunit of AChR

contains both the site for acetylcholine binding and the main targets for anti-AChR antibodies.

[0006] The autoimmune response in myasthenia gravis is directed mainly towards the extracellular domain of the AchR α-subunit (amino acids 1-210), and within it, primarily towards the main immunogenic region (MIR) encompassing amino acids 61-76 (Tzartos and Lindstrom, 1980; Tzartos et al., 1987; Loutrari et al., 1992).

[0007] The involvement of antibodies directed to the MIR and to the ligand binding site of AChR in the autoimmune process can be assessed by the ability of monoclonal antibodies (mAbs) with these specificities to passively transfer experimental autoimmune myasthenia gravis (EAMG) into animals. Examples of such antibodies are mAb 198, mAb 195, mAb 202 and mAb 35 directed towards the MIR of the extracellular portion of hAChR α-subunit (Sophianos and Tzartos, 1989), and mAb 5.5 directed towards the binding site of AChR (Mochly-Rosen and Fuchs, 1981). The anti-MIR antibodies exert their effect by crosslinking AChRs on the muscle surface thereby accelerating their degradation, and the anti-binding site mAbs by blocking and competing with acetylcholine (Souroujon et al., 1986; Asher et al., 1993; Loutrari et al., 1992a). Anti-MIR mAbs have also been shown to accelerate the degradation of AChR in the human cell line TE671 (Loutrari et al., 1992).

[0008] MG is currently treated by acetylcholinesterase inhibitors and by non-specific immunosuppressive drugs that have deleterious side effects. It would be preferable to treat MG with a method that involves antigen-specific immunotherapy but leaves the overall immune response intact. One such strategy of specific therapy could involve the administration of derivatives of AChR that do not induce myasthenia but are capable of affecting the immunopathogenic antibodies. However, since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous (Drachman, 1994), the regulation of the disease requires modulation of many antibody specificities.

[0009] Previous studies at the laboratory of the present inventors were directed towards modulating the anti-AChR response and EAMG by either denatured derivatives of Torpedo AChR, e.g. the reduced and carboxymethylated derivative, RCM-AChR (Bartfeld and Fuchs, 1978), synthetic peptides corresponding to specific regions of AChR (Souroujon et al., 1992; Souroujon et al., 1993), or mimotopes selected from an epitope library (Balass et al., 1993). The Torpedo RCM-AChR did not induce EAMG in rabbits and was effective in suppressing the disease. However, RCM-AChR did induce EAMG in rats. The experiments carried out with the synthetic peptides and mimotopes were only partially successful in neutralizing MG

autoimmune response, probably due to the incorrect folding of the short peptides that were recognized by only a portion of the anti-AChR antibodies.

[0010] MG is currently diagnosed by testing for antibodies against AChR by radioimmunoassay wherein the antigen is crude AChR extracted from human muscle or TE671 cells. This test presents some drawbacks, namely the antigen is not readily available and, in addition, the antibody titers detected are not well correlated with disease severity.

[0011] Thus, both a safe and effective treatment for MG, as well as a reliable and convenient diagnosis test, are much desired.

[0012] Oral tolerance is the phenomenon of systemic, antigen specific, immunological hyporesponsiveness that results from oral administration of antigen (Weiner, 1997). The potential of oral administration of autoantigens or their derivatives for the amelioration of autoimmune diseases was first demonstrated in a model of collagen-induced arthritis in rats that was suppressed by oral administration of type II collagen (Thompson et al., 1986 and Nagler-Anderson et al., 1986). Since then, many groups have demonstrated suppression of autoimmune responses in a variety of animal models, which led to a series of clinical trials in humans suffering from multiple sclerosis (Weiner et al., 1993), rheumatoid arthritis (Trentham et al.,

1993), uveitis (Nussenblatt et al., 1996), and type I diabetes (Schatz et al., 1996). Three basic mechanisms have been suggested to contribute to mucosal antigen-driven tolerance: clonal deletion, clonal anergy, and active suppression. These mechanisms are not mutually exclusive and may occur simultaneously to maintain stable tolerance.

[0013] Several factors are known to determine the mechanism of oral tolerance. The dose of antigen administered is the primary determinant of which mechanism predominates and may determine the outcome of oral administration of the antigen (Gregerson et al., 1993; Friedman et al., 1994 and Whitacre et al., 1991). Low doses favor active suppression, while high antigen doses favor clonal deletion and clonal anergy. For instance, oral administration of low doses (20 to 2500 μg) of type II collagen has a positive effect on rheumatoid arthritis patients, whereas larger doses did not induce active suppression of the autoimmune process and did not provide protection (Sieper et al., 1996). Similar results were also obtained in a diabetes model in mice (Bergerot et al., 1996).

[0014] Even though substantial progress has been made in elucidating the immunological mechanisms associated with antigen-specific oral tolerance, there are still many important aspects to be investigated. These include the delineation of antigen uptake and delivery in the gut, antigen processing and

presentation in the gut-associated lymphoid tissue (GALT) and costimulation requirements.

[0015] One of the open questions concerns the importance of the chemical nature of the fed tolerogen for the induction of systemic tolerance (Fowler et al., 1997). Orally administered particulate antigens often induce an active immune response, in contrast to the tolerance induced by the same antigens in soluble form (McGhee et al., 1992 and Ermak et al., 1994). The degree of nativity of the antigens is also an important issue. For instance, oral administration of type II collagen in its native form, leads to the induction of chronic autoimmune arthritis in mice, suggesting that the conformation of an orally introduced antigen could be a key factor in induction of systemic tolerance (Terato et al., 1996).

[0016] Citation of any document herein is not intended as an admission that such document is pertinent prior art, or considered material to the patentability of any claim of the present application. Any statement as to content or a date of any document is based on the information available to applicant at the time of filing and does not constitute an admission as to the correctness of such a statement.

SUMMARY OF THE INVENTION

[0017] It has now been found according to the present invention that polypeptides comprising sequences corresponding to the entire extracellular domain of the human AChR α-subunit, or to fragments thereof, are capable of modulating the autoimmune response to AChR. These polypeptides, herein referred to as "biologically active" polypeptides, were found to affect the antigenic modulation of AChR in TE671 cells *in vitro*, and to modulate the course of EAMG *in vivo*; they were effective in suppressing the disease both in EAMG that was passively transferred by monoclonal anti-AChR antibodies, and in EAMG that was actively induced by immunization with AChR, while they did not induce any symptoms of MG in the rat model system; they were further successful in both preventing EAMG and in suppressing an ongoing disease when administered nasally or orally to model rats.

[0018] Thus, the present invention provides, in one aspect, a polypeptide capable of modulating the autoimmune response of an individual to acetylcholine receptor, the polypeptide being selected from the group consisting of:

[0019] (i) a polypeptide (SEQ ID NO:6) corresponding to amino acid residues 1-210 of the human acetylcholine receptor (hAChR) α-subunit sequence depicted in Fig.1 (herein "Hα1-210"), in which is inserted, between amino acid residues 58 and

59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α-subunit gene, depicted in Fig. 2 (herein "Hα1-210+p3A");

[0020] (ii) a polypeptide (SEQ ID NO:8) corresponding to amino acid residues 1-205 of the hAChR α-subunit sequence depicted in Fig.1 (herein "Hα1-205"), in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α-subunit gene, depicted in Fig. 2 (herein "Hα1-205+p3A");

[0021] (iii) a polypeptide corresponding to amino acid residues 1-121 of the hAChR α-subunit sequence (SEQ ID NO:2) depicted in Fig.1 (herein "Hα1-121");

[0022] (iv) a polypeptide (residues 1-146 of SEQ ID NO:6) corresponding to amino acid residues 1-121 of the hAChR α-subunit sequence depicted in Fig.1, in which is inserted, between amino acid residues 58 and 59, a sequence of 25 amino acid residues encoded by the p3A exon of the hAChR α-subunit gene, depicted in Fig.2 (herein "Hα1-121+p3A");

[0023] (v) a polypeptide corresponding to amino acid residues 122-210 of the hAChR α-subunit sequence (SEQ ID NO:2) depicted in Fig.1 (herein "Hα122-210");

[0024] (vi) a polypeptide as in (i) to (v) or the polypeptide Hα1-210 (SEQ ID NO:2) in which one or more amino

acid residues have been added, deleted or substituted by other amino acid residues in a manner that the resulting polypeptide is capable of modulating the autoimmune response to acetylcholine receptor or suppressing experimental myasthenia gravis in animal models;

[0025] (vii) a fragment of a polypeptide as in (i) to (vi), which fragment is capable of modulating the autoimmune response to acetylcholine receptor or suppressing experimental myasthenia gravis in animal models;

[0026] (viii) a polypeptide comprising two or more fragments as in (vii) fused together with or without a spacer;

[0027] (ix) a polypeptide or a fragment as defined in (i)-(viii) or the polypeptide Hα1-210 (SEQ ID NO:2) fused to an additional polypeptide at its N- and/or C-termini; and

[0028] (x) soluble forms, denatured forms, chemical derivatives and salts of a polypeptide or a fragment as defined in (i)-(ix).

[0029] Preferred polypeptides according to the present invention are Hα1-121, Hα122-210 and, in particular, Hα1-210+p3A, Hα1-121+p3A, Hα1-205+p3A, optionally fused to an additional polypeptide, e.g., glutathione S-transferase (GST), and Hα1-210 similarly fused.

[0030] Preferably a fragment of Hα1-121 comprises at least the amino acid residues 61-76 of the hAChR α-subunit sequence

depicted in Fig.1, and a fragment of Hα122-210 comprises at least the amino acid residues 184-210 of the hAChR α-subunit sequence depicted in Fig.1.

[0031] In another aspect, the invention encompasses a DNA molecule coding for a biologically active polypeptide according to the invention. This DNA molecule may be selected from genomic DNA, cDNA or recombinant DNA or may be synthetically produced.

[0032] The present invention also provides a DNA molecule which includes a nucleotide sequence coding for a polypeptide of the invention, the DNA molecule being selected from the group consisting of:

[0033] (i) a DNA molecule comprising the sequence (SEQ ID NO:5) of nucleotides 1 to 630, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α-subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

[0034] (ii) a DNA molecule comprising the sequence (SEQ ID NO:7) of nucleotides 1 to 615, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α-subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

[0035] (iii) a DNA molecule comprising the sequence of nucleotides 1 to 363 of SEQ ID NO:1 depicted in Fig.1;

[0036] (iv) a DNA molecule comprising the sequence (SEQ ID NO:5) of nucleotides 1 to 363 depicted in Fig.1, in which the

sequence of the p3A exon of the hAChR α-subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175;

[0037] (v) a DNA molecule comprising the sequence of nucleotides 364 to 630 of SEQ ID NO:1 depicted in Fig.1;

[0038] (vi) DNA molecules which are degenerate, as a result of the genetic code, to the DNA sequences of (i) to (v) and which code for a polypeptide coded for by any one of the DNA sequences of (i) to (v);

[0039] (vii) a DNA molecule having a coding nucleotide sequence which is at least 70% homologous to any one of the DNA sequences of (i) to (vi) or to the DNA sequence, SEQ ID NO:1, coding for Hα1-210;

[0040] (viii) a DNA molecule as in (i) to (v) or the DNA molecule coding for Hα1-210 (amino acid sequence SEQ ID NO:2),

[0041] in which one or more codons has been added, replaced or deleted in a manner that the polypeptide coded for by the sequence is capable of modulating the autoimmune response to acetylcholine receptor or suppressing experimental myasthenia gravis in animal models;

[0042] (ix) a fragment of a DNA molecule as in (i)-(viii) which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor or suppressing experimental myasthenia gravis in animal models;

[0043] (x) a DNA molecule comprising two or more fragments of (ix) fused together with or without a spacer, and which codes for a polypeptide capable of modulating the autoimmune response to acetylcholine receptor or suppressing experimental myasthenia gravis in animal models; and

[0044] (xi) a DNA molecule comprising a nucleic acid sequence as defined in (i)-(x) or the DNA sequence, SEQ ID NO:1, coding for Hα1-210 fused to additional coding DNA sequences at its 3′ and/or 5′ end.

[0045] Preferred DNA molecules according to the invention are those comprising the sequences of nucleotides 1-363 and 364-630 of SEQ ID NO:1, depicted in Fig.1, coding for Hα1-121 and Hα122-210, respectively, and particularly the sequences of nucleotides 1-630, 1-615 and 1-363, depicted in Fig.1, in which the sequence of the p3A exon of the hAChR α-subunit gene, depicted in Fig.2, is inserted between nucleotides 174 and 175, said DNA molecules coding, respectively, for Hα1-210+p3A (SEQ ID NO:6), Hα1-205+p3A (SEQ ID NO:8) and Hα1-121+p3A (residues 1-146 of SEQ ID NO:6) that comprise the additional 25 amino acid residues coded for by the p3A exon of the hAChR α-subunit gene, as well as a DNA molecule coding for Hα1-210 fused to additional coding DNA sequences, e.g., the sequence coding for GST.

[0046] Preferably, a fragment of the DNA molecule according to the present invention codes for a polypeptide comprising at least the amino acid residues 61-76 and/or 184-210 of the hAChR α-subunit sequence (SEQ ID NO:2) depicted in Fig.1.

[0047] In still other aspects, the invention provides replicable expression vehicles comprising a DNA molecule of the invention and prokaryotic or eukaryotic host cells transformed therewith.

[0048] A further aspect of the invention relates to a process for preparation of the polypeptides of the invention comprising culturing, under conditions promoting expression, host cells transformed by replicable expression vehicles comprising the DNA molecules of the invention, and isolating the expressed polypeptides.

[0049] In yet another aspect, the present invention provides pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from the group consisting of the polypeptides of the invention and a polypeptide comprising the amino acid residues 1-210 of the hAChR α-subunit depicted in Fig. 1 (Hα1-210), soluble forms, denatured forms, salts and chemical derivatives thereof. The polypeptide Hα1-210 was previously described in the literature as a polypeptide which induces myasthenia gravis (Lennon et al., 1991), but the use of this polypeptide for

alleviation and/or treatment of myasthenia gravis is herein disclosed for the first time.

[0050] In still another aspect, the present invention provides methods for diagnosis and for alleviation and/or treatment of myasthenia gravis using the polypeptides and pharmaceutical compositions of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0051] Figure 1 depicts the nucleotide sequence (SEQ ID NO:1) and the amino acid sequence coded thereby (SEQ ID NO:2) corresponding to the extracellular domain of the hAChR α-subunit (amino acid residues 1-210).

[0052] Figure 2 depicts the nucleotide sequence (SEQ ID NO:3) and amino acid sequence coded thereby (SEQ ID NO:4) corresponding to the p3A exon of the hAChR α-subunit gene.

[0053] Figures 3A-C depict Coomassie staining (Fig. 3A) and Western blots with mAb 198 (Fig. 3B) or mAb 5.5 (Fig. 3C) of Hα1-210+p3A, Hα1-210, Hα1-121+3pA, Hα1-121 and Hα122-210 fused to glutathione S-transferase (GST) at the N-terminal (lanes 1 to 5, respectively). GST alone (lane 6) served as a control.

[0054] Figure 4 depicts results of an ELISA assay showing binding of mAb 198 to Hα1-210+p3A (filled squares), Hα1-210 (open squares), Hα1-121+p3A (filled circles) and Hα1-121 (open circles).

[0055] Figure 5 depicts results of an ELISA assay showing binding to Hα1-210+3pA of mAb 198 (filled squares), mAb 5.5 (open triangles), mAb 195 (filled "upside down" triangles), mAb 202 (filled "upright" triangles) and mAb 35 (open circles).

[0056] Figure 6 depicts results of an ELISA assay demonstrating inhibition of mAb198 (0.1µg/well) binding to AChR by the following polypeptides: Hα1-210+3pA (filled squares), Hα1-210 (open squares), Hα1-121+3pA (filled circles), Hα1-121 (open circles) and GST (filled triangles), at concentrations of 0.05-10 µg/well.

[0057] Figure 7 depicts the inhibition effect of the polypeptides of the invention on AChR degradation induced by mAb 198. TE671 cells were incubated with (a) medium, (b) 1 µg/ml mAb 198, (c-g) 1 µg/ml of mAb 198 preincubated with either Hα1-121 (hatched columns) or with Hα122-210 (dark columns) at concentrations of 10 (c), 25 (d), 50 (e), 100 (f) and 200 (g) µg/ml. Residual AChR was monitored by measuring α-bungarotoxin (α-BTX) binding sites.

[0058] Figure 8 depicts the effect of Hα1-121+p3A on AChR degradation induced by different mAbs. Residual AChR was monitored by measuring α-BTX binding sites. TE671 cells were incubated with medium alone (leftmost column) or with added mAb 198 (1 µg/ml), mAb 35 (1 µg/ml), mAb 195 (5 µg/ml) or mAb 202

(5 μg/ml) either without (dotted columns) or following preincubation of the mAbs with Hα1-121+p3A (hatched columns).

[0059] Figures 9A-B depict the effect of nasal administration of Hα1-210+p3A and Hα1-121+p3A on T cell responses to Torpedo AChR (0.25 μg/ml) (Fig. 9A), and IL-2 production in culture (Fig. 9B). Both assays were performed on cells pooled from lymph nodes taken 5 weeks after immunization with AChR from treated and control animals.

[0060] Figures 10A-B depict the effect of nasal pretreatment on the antibody titers to Hα1-210+p3A (Fig. 10A) and to rat AChR (Fig. 10B), in sera from animals treated with Hα1-210+p3A or control vehicle (GST), at 4 and 8 weeks after immunization with Torpedo AChR

[0061] Figures 11A-B depict the effect of oral pretreatment with Hα1-210+p3A and Hα1-205+p3A on the mean clinical score of EAMG (Fig. 11A) and on body weight (Fig. 11B).

[0062] Figures 12A-B depict the effect of oral pretreatment with Hα1-210+p3A and Hα1-205+p3A on T cell responses to Torpedo AChR (0.25 μg/ml) (Fig. 12A), and on the antibody titers to rat AChR (Fig. 12B).

[0063] Figures 13A-B depict the effect of oral treatment with denatured Hα1-205+p3A on an ongoing EAMG. The mean clinical score (Fig. 13A) and the mean body weight change (Fig.

13B) were monitored for 7 weeks following the beginning of treatment.

[0064] Figures 14A-C show immunochemical characterization of AChR-derived recombinant on SDS-PAGE and Western blots fragments. Torpedo AChR (5 µg; lane 1) and different recombinant fragments of human AChR α-subunit (20 µg each; GST-Hα1-210, lane 2; Trx-Hα1-210, lane 3 and Hα1-205, lane 4) were resolved on 12 % SDS-PAGE and stained by Coommassie blue (Fig. 14A) or blotted to nitrocelluse membranes that were then overlaid with ^{125}I-α-BTX (Fig. 14B) or with mAb 198 followed by ^{125}I-goat-anti-mouse (Fig. 14C).

[0065] Figure 15 shows a graph of inhibition of mAb 198 binding to Torpedo AChR by different fragments of human AChR. MAb 198 was preincubated in the presence of different concentrations of recombinant fragments and added to microtiter plates coated with Torpedo AChR. Bound mAb 198 was detected by determination of alkaline phosphatase activity.

[0066] Figure 16 shows a graph of the effect of oral treatment with recombinant fragments on ongoing EAMG.

[0067] Torpedo AChR was injected to induce EAMG and rats were treated twice a week by oral administration of OVA, Trx, Trx-Hα1-210, denTrx-Hα1-210, Hα1-205, or denHα1-205, starting eight days following AChR injection, at the acute phase of EAMG. Treatments were performed as described in Materials and

Methods section of Example 2. Representative out of three independent experiments. *P < 0.005

[0068] Figures 17A-B show bar graphs of the effect of oral administration of recombinant fragments on cytokines (Fig. 17A) and costimulatory factors (Fig. 17B). Lymph node cells from rats treated at the acute phase of EAMG with OVA (clear columns), Trx-Hα1-210 (hatched columns) or Hα1-205 (dotted columns) were cultured for 2 days in the presence of AChR, and mRNA was prepared. The mRNA expression level of cytokines or costimulatory factors (and of β-actin as control) was determined by PCR-ELISA and the data are expressed as the relative value compared to the OVA-treated group which was designated 100 %. *P < 0.005; **P<0.01

[0069] Figures 18A-B show graphs of the effects of tolerogen conformation on T and B cell proliferation. Proliferation of B and T cells from myasthenic rats in response to Torpedo AChR, Trx-Hα1-210, Hα1-205 and Trx was determined as described in Materials and Methods section of Example 2. The level of B-cell proliferation was determined by alkaline phosphatase activity (Fig. 18A) and proliferation of T-cells was determined by measuring thymidine incorporation (Fig. 18B).

DETAILED DESCRIPTION OF THE INVENTION

[0070] Patients with the neuromuscular disease myasthenia gravis are characterized by the pathogenic autoantibodies, directed towards AChR, that they develop (Aharonov et al., 1975). The α-subunit of AChR appears to be the prime target (major auto antigen) for these pathogenic autoantibodies, and within it especially the extracellular domain. Experimental autoimmune myasthenia gravis (EAMG) is also a T cell-dependent antibody-mediated autimmune disease of the neuromuscular junction in which AChR is the major autoantigen and which serves as a model for myasthenia gravis.

[0071] Human muscle AChR α-subunit exists as two isoforms consisting of 437 and 462 amino acid residues (Beeson et al., 1990). The two isoforms are identical in their amino acid composition except for a sequence of 25 additional amino acid residues inserted after position 58 in the extracellular domain of the longer variant. These additional amino acids are encoded by the 75bp exon p3A.

[0072] According to the present invention, it was found that the polypeptides herein designated Hα1-210, Hα1-210+p3A, Hα1-121, Hα1-121+p3A, Hα1-205+p3A and Hα122-210 are capable of modulating the autoimmune response to AChR and of suppressing experimental myasthenia gravis in animal models.

[0073] In order to develop an antigen-specific therapy for oral tolerance, orally or nasally recombinant fragments corresponding to the extracellular domain of the human AChR α-subunit were administered, and successful induction of protection against EAMG and suppression of an already ongoing disease were achieved. These effects on EAMG were shown to be accompanied by reduced AChR-specific cellular and humoral responses (Barchan et al., 1999 and Im et al., 1999). This is different from earlier reports in which Torpedo AChR was used for the induction of mucosal tolerance. In the latter studies, protection against EAMG was accompanied by increased anti-AChR antibody levels, probably due to the high immunogenicity of Torpedo AChR (Drachman, 1996 and Shi et al., 1998).

[0074] In order to investigate the role of tolerogen conformation for the induction of oral tolerance in myasthenia gravis, the present inventors used recombinant fragments corresponding to the extracellular domain of the human AChR α-subunit, which differ in their conformation. The different fragments were orally administered to Lewis rats during the acute phase of EAMG and their effects on disease modulation were followed. It was demonstrated that a *'more native'* fragment, Trx-Hα1-210, which is a fusion of thioredoxin and Hα1-210, failed to induce oral tolerance, whereas a *'less native'* fragment, Hα1-205, induced tolerance and was efficient

in treating ongoing EAMG. This finding was supported by the observation that these two fragments induced different changes in the cytokine profile and in the expression of costimulatory factors.

[0075] The present invention relates to the novel polypeptides Hα1-121, Hα1-121+p3A, Hα122-210, Hα1-205+p3A and Hα1-210+p3A as well as to analogs, fragments, fused derivatives (fusion polypeptides), chemical derivatives and salts thereof, and to novel analogs, fragments, fused derivatives (fusion polypeptides), chemical derivatives and salts of the peptide Hα1-210.

[0076] Analogs according to the invention are polypeptides in which one or more amino acid residues have been added to, replaced in or deleted from the original polypeptide in a manner that the resulting polypeptide retains its biological activity of suppressing experimental myasthenia gravis in animal models. Preferably, the analog is a variant of the original polypeptide or a biologically active fragment thereof which has an amino acid sequence having at least 70% identity to the amino acid sequence of the original polypeptide and retains the biological activity thereof. More preferably, such a sequence has at least 80% identity, at least 90% identity, or most preferably at least 95% identity to the native sequence. These analogs may be prepared by known synthesis procedures

and/or by genetic engineering methods, for example by expressing a DNA molecule modified by site-directed mutagenesis.

[0077] The term "sequence identity" as used herein means that the sequences are compared as follows. The sequences are aligned using Version 9 of the Genetic Computing Group's GAP (global alignment program), using the default (BLOSUM62) matrix (values -4 to +11) with a gap open penalty of -12 (for the first null of a gap) and a gap extension penalty of -4 (per each additional consecutive null in the gap). After alignment, percentage identity is calculated by expressing the number of matches as a percentage of the number of amino acids in the claimed sequence.

[0078] Analogs in accordance with the present invention may also be determined in accordance with the following procedure. Polypeptides encoded by any nucleic acid, such as DNA or RNA, which hybridize to the complement of the native DNA or RNA under highly stringent or moderately stringent conditions, as long as that polypeptide maintains the biological activity of the native sequence are also considered to be within the scope of the present invention.

[0079] Biologically active fragments of the polypeptides of the present invention are also encompassed by the present invention. As long as the fragment is capable of modulating

the autoimmune response to acetylcholine receptor and, more particularly, suppressing experimental myasthenia gravis in animal models, any fragment of Hα1-121 or Hα122-210, with or without the p3A, if the p3A site is present in the fragment, are comprehended by the present invention as long as they maintain the capability of suppressing experimental myasthenia gravis in animal models. The preferred such fragments are those which retain amino acid residues 61-76, which is the main immunogenic region (MIR) of the HACHRα subunit. A second preferred class of fragments are those which include amino acid residues 184-210 of the HACHRα subunit sequence which is the acetylcholine binding site of the HACHRα subunit. Also included in the invention are polypeptides containing two or more of such fragments which are fused together with or without a spacer.

[0080] Chemical derivatives of the polypeptides of the present invention include modifications of functional groups at side chains of the amino acid residues, or at the N- and/or C-terminal groups. Examples of such derivatives include, but are not limited to, esters of carboxyl and hydroxy groups, amides of carboxyl groups generated by reaction with ammonia or with primary or secondary amines and N-acyl derivatives of free amino groups. Cyclic forms of the polypeptides containing a disulfide bridge between two cysteines residues to stabilize

the molecule are also encompassed by the invention. Derivatives which change one amino acid to another are not encompassed by this definition.

[0081] The salts of the polypeptides of the invention are pharmaceutically acceptable, i.e., they do not destroy the biological activity of the polypeptide, do not confer toxic properties on compositions containing them and do not induce adverse effects. The term "salts" refers to salts of carboxyl groups as well as to acid addition salts of amino groups of the polypeptide molecule.

[0082] A polypeptide of the invention, or a fragment thereof, may be fused to an additional polypeptide at its N- and/or C-termini. For example, recombinant polypeptides were prepared where Hα1-210, Hα1-210+p3A, Hα1-121, Hα1-121+p3A or Hα122-210 were fused to glutathione S-transferase (GST) at the N-terminus, and these molecules were capable of suppressing the immune response to AChR. Other polypeptides may be fused to the N- and/or C-termini of a polypeptide of the invention provided that the fusion does not significantly impair the ability of the polypeptide to suppress experimental myasthemia gravis in animal models.

[0083] The results, as presented in Example 2 herein, demonstrate that when an AChR α-subunit extracellular domain polypeptide according to the present invention is fused to

another polypeptide (as fusion partner) which causes the AChR α-subunit extracellular domain polypeptide to assume a conformation which is close to its native conformation in the AChR α-subunit, such a fusion causes deleterious effects when administered nasally or orally. The best effect as a tolerogen appears to occur when the polypeptide according to the present invention is allowed to assume a conformation which is farthest from its native conformation. Indeed, from the results in Example 2, it appears that AChR α-subunit extracellular domain polypeptide *per se* functions best as a tolerogen when it is not fused to any other polypeptide. Thus, if a fusion polypeptide between an AChR α-subunit extracellular domain polypeptide, such as Hα1-120, Hα1-210+p3A, Hα1-121, Hα1-121+p3A, Hα1-205, Hα1-205+p3A, Hα122-210, etc., and another polypeptide is to be encompassed as a polypeptide according to the present invention and is to be used according to the present invention, then such a fusion polypeptide should be first tested to assure that it is not so close to the native conformation of the AChR α-subunit that it will exacerbate rather than ameliorate myasthenia gravis if administered nasally or orally.

[0084] There are several ways of testing how close any given fusion polypeptide is to the native conformation of AChR α-subunit extracellular domain. One nonlimiting example of such a test is by binding of the fusion polypeptide to αBTX. The

stronger the binding to αBTX, the more likely the AChR α-subunit extracellular domain or fragment thereof in the fusion polypeptide is close to its native conformation. Similarly, another test, in which the strength of binding of monoclonal antibody 198 to the fusion polypeptide is measured, can be used to determine how close the AChR α-subunit extracellular domain, or fragment thereof, in the fusion polypeptide is to its native conformation.

[0085] The more weakly the fusion polypeptide binds to αBTX and/or monoclonal antibody 198, the more effective the fusion polypeptide is likely to be as a tolerogen. Subsequent *in vivo* testing in the EAMG model system can be done to confirm the effectiveness of the tolerogen. Yet another test, which would instead determine fusion polypeptides which should not be used, is whether antibodies are raised when the fusion polypeptide is administered nasally or orally. If antibodies are raised after nasal or oral administration, then the fusion polypeptide is not suitable as a tolerogen.

[0086] It will be appreciated by those of skill in the art that the above tests for closeness to native conformation can also be performed on fragments, analogs and chemical derivatives of the AChR α-subunit extracellular domain to determine suitability as a tolerogen for administration to a patient suffering from myasthenia gravis. It is further

possible that while a fragment from the AChR α-subunit extracellular domain *per se* may not be considered suitable as a tolerogen, this same fragment may have its tolerogenicity improved by fusing to another peptide or polypeptide. Thus, if any given fusion polypeptide shows much weaker binding to αBTX and/or monoclonal antibody 198 relative to the fragment from the AChR α-subunit extracellular domain, then such a fusion polypeptide may be a suitable tolerogen and can be further tested for improved effectiveness in the *in vivo* EAMG model system.

[0087] A polypeptide according to the invention corresponding entirely or partially to the extracellular domain of the hAChR α-subunit should be capable of affecting the immunopathogenic response without inducing myasthenia gravis by itself. Since the anti-AChR antibody repertoire in myasthenia gravis has been shown to be polyclonal and heterogeneous (Drachman, 1994), the regulation of myasthenia gravis requires modulation of many antibody specificities. The recombinant polypeptides according to the invention have, indeed, been shown to have a broad specificity as demonstrated by their ability to protect AChR in TE671 cells against antigenic modulation induced by a series of anti-AChR mAbs (Fig 8) or by polyclonal anti-AChR antibodies from myasthenic rats (data not shown).

[0088] It was shown in several experiments (see Figs. 3B, 3C, 4 and 6) that the polypeptides comprising the additional 25 amino acid residues coded for by the exon p3A, namely Hα1-121+p3A and Hα1-210+p3A, are more potent in their protective effect in TE671 cells *in vitro* and in EAMG *in vivo*. Thus Hα1-121+p3A and Hα1-210+p3A are included along with Hα1-121 and Hα1-210 as the most preferred polypeptides according to the invention.

[0089] A polypeptide of the invention may be produced by means of recombinant technology or synthetically employing methods well-known in the art.

[0090] Recombinant polypeptides according to the invention are prepared by culturing host cells transformed by a suitable expression vector containing a DNA molecule of the invention under conditions promoting expression, and isolating the expressed polypeptide, using standard techniques well known in the art (see, for example, Sambrook et al., 1989; Ausubel et al., 1993).

[0091] Soluble forms of the polypeptides that constitute a preferred embodiment of the invention may be generated by suitable chemical modification of natural amino acid residues in the polypeptide, or by substitution of said natural amino acid residues by suitable hydrophilic natural or non-natural amino acids. Alternatively, solubility may be induced by fusion

of a polypeptide of the invention to a highly soluble polypeptide partner, such as GST, immunoglobulin or a fragment thereof, maltose binding protein (MBP), thioredoxin or influenza non-structural protein 1 (NS1).

[0092] The fused polypeptide of the invention may be used as such, or it may be subjected to further processing in which an active polypeptide of the invention is released. Insertion of a target sequence that is cleavable by specific proteases, such as V8 protease, enterokinase, thrombin or factor Xa, enables the release of the original polypeptide from the recombinant expressed fused polypeptide.

[0093] A DNA molecule according to the invention comprises a nucleotide sequence coding for a biologically active polypeptide of the invention. The DNA molecule may be from any origin including non-human sources, and may be selected from genomic DNA, cDNA, recombinant DNA, PCR-produced or synthetically produced DNA.

[0094] Preferred DNA molecules are those comprising the sequence of nucleotides 1-363 and 364-630 of the hAChR α-subunit (depicted in Fig.1) coding for Hα1-121 and Hα122-210, respectively, and particularly the sequences of nucleotides 1-630, 1-615 and 1-363 of the hAChR α-subunit in which the sequence of the p3A exon of the hAChR α-subunit gene (depicted in Fig.2) is inserted between nucleotides 174 and 175, hence

coding, respectively, for Hα1-210+p3A, Hα1-205+p3A and Hα1-121+p3A.

[0095] A fused DNA molecule according to the invention comprises a nucleic acid sequence coding for a polypeptide of the invention in fusion to additional coding DNA sequences at its 3' and/or 5' end. The added DNA sequence may code for a polypeptide endowing the expressed fused polypeptide with favorable characteristics for its purification or for performing its biological activity, i.e., conferring on the original polypeptide molecule a preferred configuration or high solubility.

[0096] A DNA molecule of the present invention may be directly isolated from human genomic DNA or cDNA by standard means known in the art involving subcloning genomic or cDNA fractions into a replicable vector, amplifying the subcloned fragments, detecting the relevant clones by their hybridization to the DNA molecules of the present invention or fragments thereof, followed by their isolation, for example as described in Sambrook et al., eds. "Molecular Cloning: A Laboratory Manual", 2nd ed., Cold Spring Harbor Press, 1989; and in "Current Protocols in Molecular Biology" Current Protocols, Ausubel et al., eds., 1993.

[0097] DNA molecules which are at least 70% homologous (sequence identity), preferably 80% homologous, more preferably

90% homologous and most preferably 95% homologous, to H1-210, Hα1-210+p3A, Hα1-205+p3A, Hα1-121, Hα1-121+p3A or Hα122-210 and encoding a polypeptide that has the biological activity of suppressing experimental myasthenia gravis in animal models may be isolated by subjecting a population of cloned genomic DNA or cDNA molecules to hybridization with the above synthesized DNA molecules or fragments thereof under stringent conditions, and isolating the hybridized clones. The term "stringent conditions" refers to hybridization and subsequent washing conditions conventionally referred to in the art as "stringent" (see Sambrook et al., 1989, and Ausubel et al., 1993).

[0098] Stringency conditions are a function of the temperature used in the hybridization experiment, the molarity of the monovalent cations and the percentage of formamide in the hybridization solution. To determine the degree of stringency involved with any given set of conditions, one first uses the equation of Meinkoth et al. (1984) for determining the stability of hybrids of 100% identity expressed as melting temperature Tm of the DNA-DNA hybrid:

$Tm = 81.5°C + 16.6\ (LogM) + 0.41\ (\%GC) - 0.61\ (\%\ form) - 500/L$

where M is the molarity of monovalent cations, %GC is the percentage of G and C nucleotides in the DNA, % form is the percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. For each 1°C that the

Tm is reduced from that calculated for a 100% identity hybrid, the amount of mismatch permitted is increased by about 1%. Thus, if the Tm used for any given hybridization experiment at the specified salt and formamide concentrations is 10°C below the Tm calculated for a 100% hybrid according to equation of Meinkoth, hybridization will occur even if there is up to about 10% mismatch.

[0099] As used herein, highly stringent conditions are those which are tolerant of up to about 15% sequence divergence, while moderately stringent conditions are those which are tolerant of up to about 20% sequence divergence. Without limitation, examples of highly stringent (12-15°C below the calculated Tm of the hybrid) and moderately (15-20°C below the calculated Tm of the hybrid) conditions use a wash solution of 2 X SSC (standard saline citrate) and 0.5% SDS at the appropriate temperature below the calculated Tm of the hybrid. The ultimate stringency of the conditions is primarily due to the washing conditions, particularly if the hybridization conditions used are those which allow less stable hybrids to form along with stable hybrids. The wash conditions at higher stringency then remove the less stable hybrids. A common hybridization condition that can be used with the highly stringent to moderately stringent wash conditions described above is hybridization in a solution of 6 X SSC (or 6 X SSPE),

5 X Denhardt's reagent, 0.5% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA at a temperature approximately 20° to 25°C below the Tm. If mixed probes are used, it is preferable to use tetramethyl ammonium chloride (TMAC) instead of SSC (Ausubel, 1993-1998).

[00100] Alternatively, a DNA molecule of the invention may be PCR-produced as described, e.g., in Example 1. In general, the PCR-production procedure comprises total RNA purification from relevant cells and generation of first strand cDNA by reverse transcriptase, using either an antisense oligonucleotide mixture or oligo (dT) as a primer. A cDNA fragment may be then amplified in a polymerase chain reaction (PCR) using appropriate sense and antisense primers flanking the target cDNA fragment. The PCR primers may include restriction sites to be used for restriction enzyme digestion followed by cloning into a suitable vector.

[00101] Cloning of a DNA molecule of the invention within an appropriate expression vehicle and expression in a suitable host cell enables production and isolation of a biologically active polypeptide or fragment thereof. For this purpose, the DNA molecule is incorporated into a plasmid or viral vector preferably capable of autonomous replication in a recipient host cell of choice. Optionally, the DNA molecule may be cloned into an expression vector in frame with additional

coding sequences at its 5′ and/or 3′ end, e.g., the pGEX plasmid vectors that contain GST coding sequences fused upstream to the cloning site. The recombinant expression vector is then used to transform an appropriate prokaryotic or eukaryotic host cell that, under inducing conditions, expresses the polypeptide itself or fused to an additional sequence. In the latter case, insertion of a recognition site for a protease, enables at will the release of the cloned polypeptide from the additional fused polypeptide.

[00102] Vectors used in prokaryotic cells include, but are not limited to, plasmids capable of replication in *E. coli*, for example, pGEX, and bacteriophage vectors such as λgt11, λgt18-23, M13 derived vectors etc.

[00103] Vectors for use in eukaryotic cells include, but are not limited to, viruses such as retroviruses and vaccinia.

[00104] A vector construct containing the DNA molecule of the invention is then introduced into an appropriate host cell by any of a variety of suitable means known in the art, such as transformation, transfection, lipofection, conjugation, protoplast fusion, electroporation, calcium phosphate precipitation, direct microinjection, etc.

[00105] Suitable host cells useful in the invention are prokaryotic cells which include, but are not limited to *E. coli*, and eukaryotic cells which include, but are not limited

to yeast cells such as *Saccharomyces cerevisiae*, or insect cell lines, for example, *Spodoptera frugiperda (Sf9)* cells, which are commonly used with the baculovirus expression system, or mammalian cells such as Chinese hamster ovary (CHO) cell lines.

[00106] Prokaryotic cells are the preferred hosts in expression systems for producing the polypeptides of the invention. Since non-native polypeptides have been shown to perform better than more native polypeptides, it is expected that polypeptides expressed in prokaryotic systems would perform better than the same polypeptides expressed in eukaryotic systems.

[00107] In another aspect, the present invention relates to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and, as active ingredient, a polypeptide selected from polypeptides of the invention, a polypeptide comprising the amino acid residues 1-210 of the hAChR α-subunit depicted in Fig.1, soluble and denatured forms, salts and chemical derivatives thereof.

[00108] The pharmaceutical compositions are for use in the alleviation and/or treatment of myasthenia gravis and may be in any suitable form for administration of polypeptides known in the art, e.g., by injection, inhalation, orally, nasally, etc.

[00109] Appropriate pharmaceutically acceptable carriers include physiological carriers, such as water and oils and excipients such as stabilizers and preservative agents. Saline solutions and aqueous dextrose and glycerol solution are suitable for injectable solutions. The active ingredient may also be prepared as a lyophilized dry compound, possibly as a salt, or as a conjugate with a solid carrier/support such as dextran, natural and modified celluloses, etc. The pharmaceutically acceptable carrier of choice will be determined depending on the route the pharmaceutical composition will be administered.

[00110] The dosage of the polypeptide and the schedule of the treatment should depend on the route of administration, the patient's condition, age and genetic background and will be determined by a skilled professional person. For example, based on animal studies, it was found that dosage ranges of about 1.4 µg - 14 mg and 0.14µg - 0.7 mg/ Kg human body weight are suitable for oral and nasal administration, respectively, in humans.

[00111] The invention further provides a method for alleviating or treating myasthenia gravis which includes administering to an individual in need thereof an effective amount of a polypeptide in accordance with the present invention.

[00112] In contrast to the current methods of treatment of MG using non-specific immunosuppressive drugs, such as steroids, azathioprine or cyclosporine, the method of the present invention is directed to an antigen-specific immunotherapy strategy which suppresses only the adverse autoimmune responses while leaving the overall immune system of the patient intact.

[00113] Preferred routes of administration of the polypeptides according to the present invention are the nasal and oral routes.

[00114] Nasal tolerization may have some advantages as a treatment modality: it requires smaller doses of toleragen, is convenient for use and does not require soybean trypsin inhibitor (STI) often used in oral tolerance to inhibit the degradation of the antigen in the gastrointestinal tract. Some successful attempts to modulate experimental autoimmune diseases in animal models by nasal administration of the autoantigen have been recently reported. Thus, Weiner et al. (1994) showed that inhalation of aerosols containing myelin basic protein (MBP) abrogated the clinical symptoms of EAE and significantly reduced the CNS inflammation, DTH reaction and antibody titer to MBP; Dick et al. (1993) reported that nasal administration of retinal extract inhibited the induction of experimental allergic uveitis (EAU) by immunization with this

extract; and Ma et al. (1995) demonstrated that nasal administration of the antigen Torpedo AChR diminished the incidence and severity of clinical muscle weakness characteristic of EAMG following immunization with the antigen.

[00115] The polypeptides of the present invention are also useful for diagnosis of myasthenia gravis whereby anti-AChR antibodies in the serum of a patient are determined by employing one or more polypeptides of the invention as the test antigen and bound anti-AChR antibody titers indicate the presence of myasthenia gravis. For the diagnostic utility, polypeptides or fusion products closest to the native conformation are preferred.

[00116] For the diagnostic test, a serum aliquot from a patient is brought in contact with one or more polypeptides, incubated for about 1 h to overnight at 4°-37°C, followed by the determination of the amount of anti-AChR antibodies bound to the polypeptides by quantitative detection assays known in the art.

[00117] In one embodiment, the diagnostic test is to be carried out with immobilized polypeptides in an assay comprising the following steps:

[00118] (i) immobilization of one or more polypeptides corresponding entirely or partially to the extracellular domain of human acetylcholine receptor on a suitable solid support;

[00119] (ii) incubation of the immobilized one or more polypeptides of step (i) with a serum sample from a patient for 1 h to overnight at 4°-37°C; and

[00120] (iii) determination of the amount of the anti-AChR antibodies bound to the immobilized polypeptides fragments, whereby detection of anti-AChR titers indicates the presence of myasthenia gravis.

[00121] The detection of the anti-AChR antibodies may be carried out with labeled anti-human antibodies or labeled *Staphylococcus* protein A. The label may be a radioactive or fluorescent tag, an enzyme conjugate or another biological recognition tag. Examples of radioactive tags are radioactive isotopes such as ^{125}I, ^{35}S, ^{32}P, ^{3}H, ^{14}C, etc, which are detected by a scintillation or a γ-counter or by autoradiography. Fluorescent tags are derived from fluorescent compounds such as fluorescein, isothiocyanate, rhodamine, phycoerythrin, phycocyanin, allophycocyanin, o-phthaldehyde and fluorescamine, and are detected by exposure of the bound fluorescent labeled antibody to light of the proper wavelength and monitoring the fluorescence.

[00122] Enzyme conjugates useful for detection purposes include, but are not limited to, maleate dehydrogenase, yeast alcohol dehydrogenase, horseradish peroxidase, alkaline phosphatase, beta-galactosidase, catalase and glucose-6-

phosphate dehydrogenase. These enzymes are conjugated to the antibody or to protein A and can be monitored by the product they produce when exposed to the appropriate substrate. The chemical moiety thus released can be detected, for example, by chemiluminescence reaction or by spectrophotometry, fluorometry or visual means.

[00123] Diagnostic methods based on recognition of biological tags include, for example, coupling of protein A or of the anti-human antibodies to biotin. The biotinylated molecules then can be detected by avidin or streptavidin coupled to a fluorescent compound, to an enzyme such as peroxidase or to a radioactive isotope and the like.

[00124] In another embodiment, the diagnostic test is carried out with one or more soluble polypeptides pre-labeled by one of the foregoing labels and tags, whereby anti-AChR antibodies of the patient's serum bound to the polypeptides are separated from the free antigen by precipitation of the antigen-antibody complex by *Staphylococcus* protein A or anti-human antibodies, and anti-AChR titers are determined as described above.

[00125] The diagnostic assays according to the invention have the advantage of avoiding the need to extract the antigen from human tissues or cells, and also provides a more reproducible and safe way for MG detection. The use as antigens

of polypeptides that recognize sub-populations of MG-related antibodies further provides a better means for correlating anti-AChR titers with disease severity.

[00126] The invention will now be illustrated by the following non-limiting examples and accompanying drawings.

EXAMPLE 1

MATERIALS AND METHODS

i) Monoclonal antibodies (mAb)

[00127] The following monoclonal antibodies were used: mAb directed towards the main immunogenic region (MIR) of the extracellular portion of the hAchR α-subunit (Sophianos and Tzartos, 1989): mAb 198, mAb 195 and mAb 202 elicited in rats against human muscle AChR, and mAb 35 elicited in rats against electric eel AChR, but cross-reacted with AChR from other species, including human; and mAb 5.5 directed towards the binding site of AChR from other species, including human (Mochly-Rosen and Fuchs, 1981), elicited in mouse against Torpedo AChR.

ii) Antibody binding assays

[00128] Binding of antibodies to AChR or to recombinant polypeptides corresponding entirely or partially to the extracellular domain of the hAChR α-subunit was analyzed by

ELISA. Wells of microtiter plates (Maxisorb, Nunc, Neptune, NJ) were coated by incubation overnight at 4°C with either Torpedo AChR (1µg in 100 µl of phosphate-buffered saline (PBS)), or with one of the recombinant polypeptides of the invention (2µg in 100 µl of 50 mM Tris buffer pH 8.0). Coated plates were washed three times with PBS containing 0.05% Tween-20, then wells were blocked by incubation for 1 h at room temperature (R.T.) with 1% bovine serum albumine (BSA) and 1% hemoglobin in PBS, and the coated blocked plates were then washed and incubated overnight at 4°C with different amounts of antibody.

[00129] For inhibition experiments, each well was coated with 1 µg of Torpedo AChR and a polypeptide of the invention was preincubated with the mAb of choice for 30 min at R.T. before addition to the AChR-coated well. Following a washing step, bound mAb was determined by incubation for 1 h at R.T. with 1:5000 dilution of alkaline phosphatase (AP)-conjugated goat anti-mouse Igs (Jackson ImmunoResearch Labs, Inc., or Biomakor, Ness-Ziona, Israel). The bound antibody was detected by the enzymatic activity of AP using N-para-nitrophenyl-phosphate as a substrate and determining by a microtiter plate reader at 405 nm the color developed after about 40 min.